

REMARKS

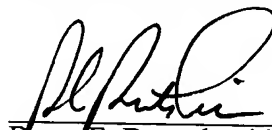
Applicants have amended the specification and claims to put this application in better condition for examination in the United States. The additions to the application include a Brief Description of the Drawings, an Abstract of the Disclosure based on the abstract accompanying the published International Application and claims directed to aspects of the invention disclosed but not claimed in the International Application as filed. These amendments introduce no new matter into this application.

Early action allowing claims 4-12 is solicited.

Attached hereto is a marked-up version of the changes made to the specification by this amendment, captioned "Version marked to show changes made".

In the event that the transmittal letter is separated from this document and the Patent and Trademark Office determines that an extension and/or other relief is required, applicants petition for any required relief including extensions of time and authorize the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing 491332000300.

Respectfully submitted,



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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Please amend the specification as follows:

SYNTHESIS OF A NOVEL PARAMAGNETIC AMINO ACID DERIVATIVE (EPM-5) FOR LABELING DIFFERENT MACROMOLECULES AND SYSTEMS OF CHEMICAL-BIOLOGICAL INTEREST

FIELD OF THE INVENTION

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This invention ~~refers~~ relates to the chemical synthesis of a new paramagnetic β -amino acid derivative containing ~~the~~ a stable nitroxide radical moiety inserted in its pyrrolidine structure and in which the 9-fluorenylmethyloxycarbonyl group (Fmoc) was chosen as its amine function protecting group. This paramagnetic compound is ~~therefore~~ a new type of spin probe (or spin label) and it can be used as alternative report molecule for labeling peptide sequences, other macromolecules and systems where ~~the~~ electron paramagnetic resonance spectroscopy (EPR) can be applied. ~~The use of this~~ This compound can be ~~extended~~ used also for other spectroscopic methods such as fluorescence and nuclear magnetic resonance since its paramagnetism may affect the spectra of ~~this~~ these methodologies. Due to the presence of both carboxyl and amine groups in its structure, ~~the use of this organic compound may be extended~~ used for labeling a great variety of other molecules or systems containing reactive ~~function~~ functions for these two groups.

BACKGROUND AND SUMMARY OF THE INVENTION

The intermediate for the synthesis of the amino acid derivative of ~~the present patent~~ this invention contains the structure 2,2,5,5-tetramethylpyrrolidine-1-~~oxil~~ oxyl-3-amine-4-carboxylic acid, henceforth denominated as Poac, synthesized more than two decades ago (~~v.g.~~ (see, Tetrahedron 491-499 [1965] and Bull. France, 3, 815-817 [1967])). ~~Thus, the~~ The Poac derivative containing the Fmoc protecting group (2,2,5,5-tetramethylpyrrolidine-1-~~oxil~~-3-(9-

fluorenylmethyloxycarbonyl) oxyl-3-(9-fluorenylmethyloxycarbonyl)-amine-4-carboxylic acid) is the novel spin probe derivative of the present ~~patent of invention~~ **invention, which also includes the synthesis and use in EPR of this spin probe derivative**. This new compound allows the ~~POAC~~ insertion **of Poac** as ~~an a~~ usual amino acid at any position of ~~the a~~ peptide sequence, and its denomination will be Fmoc-Poac or EPM-5 in this ~~descriptive report~~ **application**. The chemical structure of this paramagnetic molecule is represented in Figure 1.

~~The electron~~ **Electron** paramagnetic resonance (EPR) [**described** in *Biological Magnetic Resonance*, Berliner, L. J. and Reuben, J., eds., Plenum Publishing, New York, (1989)], is a modern and very useful spectroscopic method because it allows ~~studies~~ **the study** of any paramagnetically labeled macromolecules or biological systems regarding their conformation, mobilities, inter- or intra-molecular interactions, structuring ~~state, etc~~ **states, and the like**. The wide spectrum of EPR application is already detailed in the literature (~~v.g.~~ **(see, Free Nitroxyl Radical**, Rozantsev, E.G., Ulrich, H., ed., Plenum Press, London, 1970), where a great variety of spin labels, i.e., chemical compounds which are paramagnetic due to the presence of an unpaired electron in ~~its~~ **their** structure, is listed. They are, therefore, a class of free ~~radical~~ **radicals** but ~~must be~~ **are** necessarily stable ~~towards~~ **under conditions around** normal temperature and physiological pH and also allow several chemical reactions or experiments without affecting ~~its~~ **their** free radical moiety.

Amongst the most commonly used spin labels one can ~~detach~~ **pick out** the nitroxide group-containing molecules and where the unpaired electron locates. The most significant progress in the ~~RPE~~ **EPR** field for labeling of relevant biological structures such as peptides and proteins was achieved with this class of spin probes. Almost two decades ago appeared the first ~~RPE~~ **EPR** application in the solid phase peptide synthesis methodology [(*The Peptides: Analysis, Synthesis and Biology*, vol. 2, Academic Press, New York, (1980)]. This approach was introduced by our group using, instead, ~~an other~~ **another** nitroxide-containing spin label,

denominated at that time as Toac (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amine-4-carboxylic acid), ~~which~~ **which** protected at it ~~its~~ **its** amine function with the acid labile tert-butylloxycarbonyl ~~butyloxycarbonyl~~ **butyloxycarbonyl** (Boc) group [~~v.g. (see,~~ **(see,** Braz. J. Med. Biol. Res. 14, 173, (1981) and Biochim. Biophys. Acta, 742, 63, (1983)]. Thus the Boc-Toac spin probe was the first in the literature used to label a peptide sequence as an amino acid. However, due to chemical particularities of the peptide synthesis methodology, it was only possible to couple the Toac group at the peptide N-terminal position. To overcome this shortcoming, an alternative strategy was published by us which finally allowed the insertion of the spin label internally to the peptide sequence [~~v.g. see,~~ **see,** J. Am Chem. Soc. ~~115~~ **Soc. 115**, 11042 (1993)].

An impressive increase in the application of ~~RPE~~ **EPR** for peptide chemistry field was further observed ~~with in~~ **in** reports investigating peptide conformational properties [v.g. J. Am. Chem. Soc. 117, 10555 (1995); FEBS Lett. 375, 239 (1995); Biopolymers 42, 821 (1997)] or of ~~peptidil~~ **peptidyl**-resin solvation (Tetrahedron Lett. 375, 239 [1995]; Biopolymers 42, 821 [1997]). As an amino acid, ~~TOAC~~ **Toac** was introduced in different positions of some biologically active peptides such as angiotensin II and bradykinin, but a partial or ~~integral~~ **total** loss of their biological potencies were observed due to the introduction of a non natural compound in their structures. [*Peptides 1996*, R. Ramage and R. Epton, eds., Mayflower Scientific Co. p. 673; (1998)].

However, we recently described the synthesis of a peptide hormone labeled with Toac and where its biological potency was entirely preserved. [~~v.g. e.g.,~~ **e.g.,** FEBS Lett. 446, 45 (1999)]. This result was obtained with the tridecapeptide α -melanocyte stimulating hormone analogue and, owing to its potentialities in a great number of chemical-biological assays (this analogue is paramagnetic, naturally fluorescent and fully active), ~~was submitted for the patent process (PI 9900595, February 24, 1999: "Synthesis of the first paramagnetic and active melanocyte stimulating hormone analogue containing free radical amino acid")~~, **and was disclosed in**

commonly owned U.S. patent application Serial No. 09/935, 760, entitled "Paramagnetic And Active Analogue (EPM-2) Of Melanocyte Stimulating Hormone Containing Amino Acid-Type Stable Free Radical").

In spite of these results, one still remaining shortcoming to in the use of Toac in peptide chemistry refers to is the severe difficulty in coupling the subsequent amino acid residue of the peptide sequence during the synthesis. ~~It~~ This difficulty seems to be due to the low nucleophilicity of the Toac amine group, whose pKa of 8 (when in free state) decreases to about 5.5 when bound to the N-terminal portion of a peptide chain [v.g., Braz. J. Med. Biol. Res. 14, 173 (1981) and Biochim. Biophys. Acta. 742, 63 (1983)]. Several recouplings and ~~the~~ an increase on in the temperature of the coupling reaction are usually necessary to assure complete incorporation of the subsequent amino acid of the desired peptide sequence [J. Am. Chem. Soc. 115, 11042 (1993)]. Soc. 115, 11042 (1993)].

Aiming to overcome this limitation inherent to the Toac probe and searching for BRIEF

DESCRIPTION OF THE DRAWINGS

Figure 1 is a depiction of the structure of Fmoc-Poac or EPM-5 according to this invention.

Figure 2 is a mass spectrum of Poac.

Figure 3 is a high performance liquid chromatography (HPLC) profile of Poac showing a single peak.

Figures 4A and 4B depict EPR spectra of Poac in water and Fmoc-Poac in dimethylformamide, respectively.

Figure 5 is a mass spectrum of Fmoc-Poac.

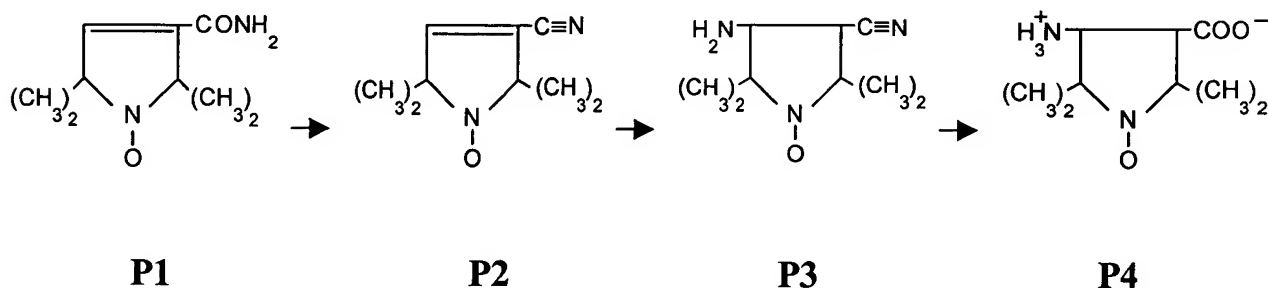
Figure 6 is a HPLC profile of Poac⁷ showing a single peak.

Figure 7 is a mass spectrum of Poac⁷-angiotensin II.

Figures 8A, 8B and 8C depict EPR spectra of Poac⁷-angiotensin II at pH 3.0, 6.0 and 9.0, respectively.

DETAILED DESCRIPTION OF THE INVENTION

In order to overcome the limitations of the Toac probe to find an alternative spin label which may induce a differentiated conformational constraints in peptide structures, we decided to synthesize the FMOC POAC according to partially described in accordance with this invention, we synthesized Fmoc-Poac according to the synthetic route shown below {that is partially described in Tetrahedron, 491-499 (1965); Bull. Soc..Chim. France, 3, 815 (1967)}:



Step #1 - Synthesis of 2,2,5,5-tetramethylpyrroline-1-oxyl-3-carbonitrile (P2)

This product was synthesized by treating the compound P1 (from Sigma Co) with tosyl-tosyl chloride in dry pyridine. To 28.7 g (1.5×10^{-1} mol) of tosyl-tosyl chloride, 15.3 g (8.35×10^{-2} mol) of P1 dissolved in 100 mL of dry pyridine was added and left at room temperature for 48 hours. After this period, 10 g of KOH dissolved in 250 mL of water were added, and the mixture was heated up for to 80°C. After cooling, the product was extracted with sulfuric ether, washed with diluted HCl, diluted NaHCO₃ solution, water and dried over anhydrous Na₂SO₄. After evaporation of the solvent under reduced pressure 12.76 g (yield= 92%) of an orange powder was obtained and further purified in an alumina column using

benzene as an eluent. The product (P2) showed a single spot in thin layer chromatography and with following characteristics: M.P.= 62-63°C, M+ = 165; Elementary analysis ÷ found: C, 65.36% , H, 7.50% e N, 17.10 % ; calculated : C, 65.43% , H, 7.93% e N, 16.96%).

Step #2 : Synthesis of 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-amine-4-cyano (P3).

In a 3 L round bottom flask, 600 mL of liquid ammonia, 9 g (5.44×10^{-2} mol) of P2 and 120 mL of water were added. The flask was tightly closed to maintain the mixture under pressure and left at room temperature. After 3 days, the ammonia was eliminated and the product extracted with chloroform. The crude product crystallized from ether-petroleum ether yielded 9.37 g (yield = 94%) of a yellow powder with the following characteristics: M+= 182; P.F. 84-85 °C; Elementary analysis ÷ found: C 58.98%; H, 8.37%; N, 22.21%; calculated (C₉H₁₆ON₃): C, 59. 31%; H, 8.85 % ; N, 23.06 %).

Step 3 - Synthesis of 2,2,5,5-tetramethylpyrrolidine-1-oxil tetramethylpyrrolidine-1-oxyl-3-amino-4-carboxylic-acid (P4) -POAC(Poac).

8 g (4.38×10^{-2} mol) of P3 and 40 g of Ba(OH)₂ were suspended in 600 mL of water and added to a 3 L round bottom flask. The flask was tightly closed and heated up to 120°C for 90 minutes. After cooling, the mixture was neutralized with an excess of dry ice and filtered. The aqueous solution was concentrated under reduced pressure and yielded 8 g of crude product (yield: 90%) that was crystallized from 90% ethanol. The product presented the following characteristics: M.P. = 212°C (melts with sublimation); M+ = 201 (Figure 2), single ~~piek~~ peak in HPLC; (Figure 3); elementary analysis ÷ found : C, 53.1% ; H, 8.28 %; N, 13.95 % ; calculated (C₉H₁₇N₂O₃): C, 53.71 % ; H, 8.52; N, 13.92 Infra-red (KBr) : cm⁻¹ : 3084, 2872, 2792, 2548 and 2132 (NH+3); 1643 ν_{AS} (NH+3) ; 1574 (ν_{AS} , C=O); 1456 (δ CH₃); 1396 and 1376 (gem-dimethyl and COO-); 782 (δ C=O). The Figure 4A shows the EPR spectrum of POAC in aqueous solution, pH 5. The calculated values for the two rotational correlation times (τ_B and τ_c) were 0.509×10^{-10} and 0.597×10^{-10} , respectively.

Preparation of Fmoc-Poac

201 mg (1 mmol) of P4 was dissolved in 1.5 mL of water in presence of 286 mg of sodium carbonate. ~~10 H₂O~~ carbonate.10H₂O in which 337 mg (~~1mmol~~) (1 mmol) of Fmoc-succinimidyl-carbonate dissolved in 1.5 mL of acetone was added drop-wise. The reaction was carried out at room temperature with stirring, and the pH was maintained around 9 by addition of sodium carbonate. After 3 hours, the mixture was diluted with 25 mL of water, and acidified with 1 N HCl until it reached pH 2. The desired product was extracted with ethyl acetate, washed with small portions of water, dried over anhydrous sodium sulfate. After filtration, the solvent was evaporated under reduced pressure. The crude product was crystallized twice with chloroform and yielded 380 mg (yield : 90%). The product was characterized by mass and infrared spectroscopy, elementary analysis and EPR. Characteristics: M⁺ = 423 (Figure 5); elementary analysis : found: C, 67.9 % ; H, 6.35 %; N, 6.60 %; calculated (C₂₄H₂₇O₅N₂): C, 68.08 % ; H, 6.28 % ; N, 6.62%; IR (KBr) cm⁻¹: 3444-3338 (broad band OH and -CONHR); ~3030 (ν_{Ar} CH); 3000-2700 (ν_{Ar} COOH); 1723 (R-O-C-ON- and COOH); 1543 (δ_{NH} and ν_{CN}); 1450 (δ CH₃); 1235-1150 (gem-dimethyl group). The EPR spectra of Fmoc-POAC Poac in dimethylformamide is represented in Figure 4B and the calculated τ_B and τ_c values are 1.14 x 10⁻¹⁰ s.rad⁻¹ and 1.79 x 10⁻¹⁰ s. rad⁻¹, respectively.

Synthesis of Poac⁷-angiotensin II

Angiotensin II analogue labeled with the spin probe POAC Poac (Asp-Arg-Val-Tyr-Ile-His-Poac-Phe) was synthesized in 0.15 mmol scale, by the solid phase method already mentioned and with alteration to provide the insertion of this marker in the middle of the peptide chain. [J. Am. Chem. Soc. 115, 11042 (1993)]. Fmoc Fmoc-Phe-Wang-resin [J. Am. Chem. Soc. 95, 1328 (1973)] with 0.41 mmol/g substitution degree acquired commercially was used. All couplings were carried out using 2.5 fold excess for the Fmoc Fmoc-amino acids and 3 fold excess for Fmoc-POAC Poac. The acylating reagents for coupling were

diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole in hydroxybenzotriazole in a dichloromethane:dimethylformamide mixture (1:1, v/v) as a solvent. The Fmoc deprotection was performed with 20% piperidine in dimethylformamide (v/v) for ~~20min~~ 20 min.

Interestingly, this synthesis demonstrated that the pyrrolidine structure of the Poac spin label allowed much easier incorporation of the subsequent amino acid residue than the has been observed during the Toac⁷-AII synthesis. In this latter case, repetitive recoupling reactions were necessary, including the an increase in the temperature. These procedures were not necessary in the case of the Poac ~~derivative~~ derivatives, thus demonstrating that the Poac amine group reactivity is much higher than that of Toac.

After the completion of the synthesis, the peptide was cleaved from the resin in anhydrous HF containing 10% of the a p-cresol and dimethylsulfide mixture, at 0°C for 90 min. The crude peptide obtained after extraction and lyophilization (125 mg) was dissolved in 70 mL of water and the pH was raised to 10 with ammonium hydroxide and stirred for 2 h, to ~~revert~~ reverse the nitroxide protonation that occurs during the HF treatment. After lyophilization, the peptide was purified by preparative HPLC (high performance liquid chromatography) using a reverse phase C₁₈ (25 x 250 mm) and ammonium acetate 0.02 M, pH 5 and acetonitrile 60% in water, as solvents A and B, respectively. The linear gradient applied was from 20-65% of B in for 135 min.

The homogeneity of the peptide was confirmed through analytical HPLC (Figure 6), mass spectrometry, M⁺ = 1132.55 (Figure 7), and the amino acids analysis showed the expected composition : Asp 0.95 (1.00); Val 0.96 (1.00); Ile 1.20 (1.00); Tyr 1.02 (1.00); Phe 1.00 (1.00); His 0.96 (1.00); Arg 1.02 (1.00). ~~The~~ Figure 8 displays the EPR spectra of 0.25 mM Poac⁷-AII at pH 3, 6 and 9 aqueous solution. No significant variation on the rotational correlation time values was observed for this paramagnetic AII analogue thus suggesting that its ~~conformations~~ conformation is not affected by the pH of the media.

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